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Prenatal zinc deficiency-dependent epigenetic alterations of mouse metallothionein-2 gene☆

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Abstract

Zinc (Zn) deficiency *in utero* has been shown to cause a variety of disease states in children in developing countries, which prompted us to formulate the hypothesis that fetal epigenetic alterations are induced by zinc deficiency *in utero*. Focusing on metallothionein (MT), a protein that contributes to Zn transport and homeostasis, we studied whether and how the prenatal Zn status affects gene expression. Pregnant mice were fed low-Zn (IU-LZ, 5.0 μg Zn/g) or control (IU-CZ, 35 μg Zn/g) diet *ad libitum* from gestation day 8 until delivery, with a regular diet thereafter. Bisulfite genomic sequencing for DNA methylation and chromatin immunoprecipitation assay for histone modifications were performed on the *MT2* promoter region. We found that 5-week-old IU-LZ mice administered cadmium (Cd) (5.0 mg/kg b.w.) have an elevated abundance of *MT2* mRNA compared with IU-CZ mice. Alteration of histone modifications in the *MT2* promoter region having metal responsive elements (MREs) was observed in 1-day-old and 5-week-old IU-LZ mice compared with IU-CZ mice. In addition, prolongation of MTF1 binding to the *MT2* promoter region in 5-week-old IU-LZ mice upon Cd exposure is considered to contribute to the enhanced MT2 induction. In conclusion, we found for the first time that Zn deficiency *in utero* induces fetal epigenetic alterations and that these changes are being stored as an epigenetic memory until adulthood.

Keywords: DNA methylation; Epigenetics; Histone modification; Metallothionein; Zinc

1. Introduction

The malnutritional status *in utero* has been shown to affect the progeny's health and disease states later in life in humans as well as in laboratory animals. Low-birth-weight babies resulted from prenatal malnutrition can be a risk factor for lifestyle-related diseases, such as ischemic heart disease and diabetes [1–4]. This hypothesis has been widely acknowledged and expanded to the concept, named 'Developmental origins of health and disease (DOHaD)' [5]. Recently, rats that were grown under low-protein nutritional conditions *in utero*, or had intrauterine growth retardation have been shown to develop hypertension or type 2 diabetes later in adulthood [6–9]. Moreover, a plethora of published works have shown that extrinsic conditions *in utero*, such as nutrition and environmental chemicals, affect the propensity of the fetus to develop disease states later in adulthood [10–12]. Although the underlying mechanism is still under intensive investigation, there is a widely-acknowledged view that epigenetic alterations, namely, DNA methylation (the covalent addition of a methyl group to the 5′-carbon of cytosine in the CpG dinucleotide) and histone modification (methylation, acetylation, phosphorylation, ADP-ribosylation, and ubiquitination), play a pivotal role in the expression of particular genes, which will subsequently alter the physiological status of the whole organism. Gene expression is suppressed by DNA methylation of the promoter region of a given gene [13], whereas histone modifications regulate chromatin structure and alter gene activity [14]. Such epigenetic alterations could be inherited by succeeding generations [15].

Experimentally, a few studies have shown that zinc (Zn) restriction during pregnancy induces disease states later in life. Rats grown under prenatal or postnatal Zn restriction have been reported to develop hypertension [16] and impairments of learning and memory [17,18] later in adulthood. Pregnant mice fed a Zn-deficient diet *in utero* have shown persistent immunodeficiency for three succeeding generations [19]. Zn is an essential trace element and a key component of approximately 300 enzymes in various types of tissues [20,21]. Zn deficiency induces various disease states in humans, such as immunodeficiency, developmental disorders, alopecia, dysgeusia, skin disorders and anemia. Vegetarians [22], elderly

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persons [23], habitual alcohol drinkers [24], infants and pregnant/parturient women [22] have the tendency to develop Zn deficiency. In addition, maternal Zn deficiency during pregnancy induces pregnancy complications, delayed delivery, and low-body-weight birth [25]. Zn deficiency is responsible for 4.4% of deaths of children aged 6–59 months in developing countries [26].

Metallothionein (MT), a low-molecular weight protein, has been shown to be involved in the transport, metabolism and homeostasis of heavy metal ions, such as Zn and copper, in tissues and cells. One-third of their amino acid residues of this protein are cysteine residues without a disulfide bond. This characteristic enables MTs to play a role in the transport and inactivation/detoxification of metals [27,28]. Aside from the metabolism and homeostasis of heavy metals, MT has been known to protect cells from oxidative stress and inflammation elicited by various environmental stimuli including heavy metals. Among the four MT isoforms known so far, MT1 and MT2 exist in nearly all types of cells in the body. It has been established that the expression of *MT1/2* genes is induced by metal ions, such as Cd, Zn, Cu and Hg [27]. For the up-regulation of *MT1/2* transcription upon exposure to these metal ions, metal responsive elements (MREs) located in the promoter region are essential. A limited line of experimental evidence showed that metal transcription factor 1 (MTF1) [29] will bind to the MRE motif upon exposure to at least Zn ions, and the Zn-ion-bound MTF1 forms a complex with p300 and Sp1, and then this complex is recruited to MREs of the *MT1* promoter region [30].

The effects of prenatal zinc deficiency on MT regulation have been studied. Pregnant mice were fed either a control diet (100 μg Zn/g) or a low-Zn diet (5.0 μg Zn/g) from gestation day 7 to delivery, and both groups of dams were given the control diet after delivery. Although the Zn and MT levels in pups born to these two groups of dams were similar at postnatal day 3, serum IgM concentrations were significantly lower in adulthood in the mouse offspring born to dams given the low-Zn diet than in the offspring born to control dams. Moreover, when the mouse offspring was given Zn injections to stimulate MT synthesis, the mice deprived of Zn while *in utero* had markedly higher MT levels in the liver than control mice later in adulthood [31].

Collectively, prenatal Zn deficiency has been shown to induce disease states, which is presumably due to epigenetic alterations. However, nearly no studies to elucidate the molecular basis of disease states induced by prenatal Zn deficiency are available. Thus, we have hypothesized that fetal epigenetic alterations can be induced by Zn deficiency *in utero* and alter the physiological conditions that will lead to the onset of disease conditions later in adulthood. In this study, we developed an experimental animal model of prenatal Zn deficiency and studied whether Zn deficiency *in utero* exerts fetal epigenetic alterations in *MT1/2* genes.

2. Materials and methods

2.1. Reagents

The following reagents were purchased from the manufacturers described in parentheses: RNase A, mouse monoclonal anti-β-actin IgG1 and CellLyticNuCLEARExtraction kit (Sigma-Aldrich, St Louis, MO, USA); RNeasy Mini kit, QIAquick PCR Purification kit, QIAquick Gel Extraction kit and QIAprep Spin Miniprep kit (Qiagen K.K., Tokyo, Japan); Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA); Wizard DNA Clean-Up system, pGEM-T Easy Vector, pGL4.0 Luciferase Reporter Vector, phRL-TK Vector and Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA); proteinA agarose/salmon sperm DNA, rabbit polyclonal anti-acetylated histone H3 IgG, rabbit polyclonal anti-acetylated histone H4 IgG, rabbit polyclonal anti-acetylated histone H3 lysine14 IgG and Immobilon-P transfer membrane (MilliPore, Billerica, MA, USA); proteinase K, Blocking One and Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan, USA). *Bam* HI, *Aci* I, *Kpn* I, *Xho* I and *Dpn* I were purchased from New England Biolabs Japan (Tokyo, Japan); rabbit polyclonal anti-MTF1 IgG and goat polyclonal anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA); Immunopure goat anti-rabbit IgG, F(ab′)2, peroxidase conjugated, ImmunoPure goat anti-mouse IgG, F(ab′)2, peroxidase conjugated and Immuno Pure rabbit anti-goat IgG, F(ab′)2, peroxidase conjugated (Thermo Fisher Scientific, Rockford, IL, USA); rabbit polyclonal anti-acetylated histone H3 lysine9 IgG and rabbit polyclonal anti-trimethylated histone H3 lysine4 IgG (Cell Signaling Technology, Danvers, MA, USA). LightCycler480 SYBR Green I Master (Roche Diagnostics Japan, Tokyo, Japan); Minisart SRP 15 (Sartorius Stedim Biotech, Goettingen, Germany). IGEPAL-CA630 (Wako Pure Chemical, Osaka, Japan); PrimeScript RT reagent kit, SYBR Premix Ex Taq, TaKaRa Ex Taq, LA Taq and T4 polynucleotide kinase (TaKaRa BIO, Otsu, Japan). *Not* I, DH5α and KOD -Plus (Toyobo, Osaka, Japan); Ligation Convenience kit and ISOGEN (Nippon Gene, Tokyo, Japan); all other reagents of analytical grade (Sigma-Aldrich, Invitrogen and Wako Pure Chemical); all oligonucleotides (Hokkaido System Science, Sapporo, Japan).

2.2. Animals

C57BL/6J strain pregnant ($n=44$) and male mice ($n=18$) were purchased from CLEA Japan. The mice were housed in a room with temperature at 23±1°C and humidity at 50±10% on a 12/12-h light–dark cycle. We used three kinds of rodent chow. Laboratory rodent chow (50 μg Zn/g; Labo MR Stock, Nosan) was given to mice unless specifically described. Low-Zn diet (5.0 μg Zn/g) or control diet (35 μg Zn/g) (CLEA Japan) was used in Zn-deficiency experiments. According to the previous studies [32,33], zinc concentration in diet (35 μg Zn/g) was found to be high enough to be used for a control diet group. These chows and deionized water were provided *ad libitum*. For this study, male mice were used unless specifically described. The experiments protocols using mice were approved by the Animal Care and Use Committee of the Graduate School of Medicine, the University of Tokyo.

2.3. Experiments on Zn deficiency in utero

Pregnant mice were fed a Labo MR Stock rodent chow until gestation day 7, and the chow was replaced with a low-Zn diet or a control diet thereafter until delivery. On the day of birth, two to three male pups per dam were randomly adopted from fourteen dams to minimize possible litter effects and to make two groups: (1) *in utero* low-Zn (IU-LZ) mice and (2) *in utero* control (IU-CZ) mice. The pups were decapitated by scissors, and their livers were harvested. All the liver tissues except those used for chromatin immunoprecipitation (ChIP) assay were immediately frozen in liquid nitrogen, and kept at −80°C until analyses. Livers used for the ChIP assay were immediately minced by scissors and subjected to the subsequent processes as described in the ChIP assay section below. The number of pups for each dam was adjusted to be 6 to 7 pups by adoption from other dams on the day of birth. The dams were given Labo MR Stock rodent chow from the delivery to weaning. After weaning, male pups were given Labo MR Stock rodent chow thereafter.

When IU-CZ mice and IU-LZ mice became 5 weeks old, they were administered orally with a single dose of cadmium (Cd) (5.0 mg kg^{-1} b.w.). Mice were sacrificed by cervical dislocation, and livers were harvested 0, 1 and 6 h post Cd administration (Fig. 1A).

2.4. Experiment on Zn deficiency in adulthood

Male mice aged 10 weeks were fed a low-Zn(AD-LZ) diet or a control (AD-CZ) diet *ad libitum* for 12 days, and then sacrificed by cervical dislocation to harvest the liver. Other mice were fed Labo MR Stock rodent chow for another 30 days, and administered orally a single dose of Cd (5.0 mg kg^{-1} b.w.). Livers were collected 6 h after Cd administration (Fig. 1B).

2.5. Measurement of Zn and Cd concentrations

Livers (approx. 0.1 g) and blood (approx. 0.2 g) specimens were digested in 2 ml of concentrated nitric acid in glass test tubes. The temperatures were kept at 80°C for 1 h, with a gradual increase with 10°C for 1 h each to 130°C. When the acid-digested specimens were became transparent, they were diluted with 1% $HNO_3$and filtered with Minisart SRP 15 and determined for Zn and Cd concentrations by inductively coupled plasma mass spectrometer (Agilent 7500ce; Agilent Technologies).

2.6. RNA isolation and reverse transcription

Total RNA was isolated using RNeasy Mini Kit and then reverse-transcribed using PrimeScript RT reagent Kit, according to the manufacturer's instructions.

2.7. DNA isolation

DNA was isolated using ISOGEN, according to the manufacturer's instructions and purified by phenol/chloroform extraction method.

2.8. Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) analysis was performed using SYBR Premix Ex Taq and amplified by LightCycler under the following conditions: 95°C/10 s ×1 cycle; 95°C/5 s, 60°C/30 s, ×45 cycles or using LightCycler480 SYBR Green I Master and amplified by LightCycler480 under the following conditions: 95°C/5 min ×1 cycle; 95°C/15 s, 60°C/10 s, 72°C/30 s ×45 cycles. Primers used in the qPCR analysis